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(54) Title: REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY			
(57) Abstract			
<p>This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS and nNOSμ). According to a first aspect, the invention provides a method of identifying modulators of AMPK-mediated activation of eNOS, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations. In an alternative aspect, the invention provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably specific phosphorylation of threonine 495 is assessed. According to a second aspect, the invention provides a method of identifying modulators that either promote or inhibit phosphorylation of nNOS and nNOSμ at Ser-1417. Compounds which activate the AMP-activated protein kinase are expected to be useful in the treatment of ischaemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds would also have utility in the treatment of pulmonary hypertension and in obstructive airways disease.</p>			

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1 REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY

2 This invention relates to the regulation of the
3 activity of the enzyme nitric oxide synthase, and in
4 particular to regulation of activity of endothelial and
5 neuronal nitric oxide synthases. We have found that the
6 phosphorylation of endothelial and neuronal nitric oxide
7 synthases by several protein kinases, including protein
8 kinase C and the AMP-activated protein kinase, regulates
9 their activity .

10

11 BACKGROUND OF THE INVENTION

12 Nitric oxide (NO) has recently been recognised as
13 an important mediator of a very wide variety of cellular
14 functions, and is present in most if not all mammalian
15 cells (Moncada, S. and Higgs, A., 1993). It is implicated
16 in a range of disorders, hypertension,
17 hypocholesterolaemia, diabetes, heart failure, aging,
18 inflammation, and the effects of cigarette smoking, and is
19 especially important in vascular biology. It regulates
20 systemic blood pressure as well as vascular remodelling
21 (Rudic et al., 1998) and angiogenesis in response to tissue
22 ischaemia (Murohara et al., 1998). NO is synthesised from
23 the amino acid L-arginine by the enzyme nitric oxide
24 synthase (NOS).

25 Three isoforms of NOS have been identified:
26 neuronal NOS (nNOS), which is found in neuronal tissues and
27 skeletal muscle (nNOS μ isoform); inducible NOS (iNOS),
28 found in a very wide variety of mammalian tissues including
29 activated macrophages, cardiac myocytes, glial cells and
30 vascular smooth muscle cells; and endothelial NOS (eNOS),
31 found in vascular endothelium, cardiac myocytes and blood
32 platelets. Endothelial cells produce NO in response to

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1 shear stress generated by the streaming of blood on the
2 endothelial layer.

3 The three isoforms of NO synthase have an amino
4 acid sequence identity of approximately 55%, with strong
5 sequence conservation in regions involved in catalysis.
6 For all three isoforms, the mechanism of NO synthesis
7 involves binding of the ubiquitous calcium regulatory
8 protein calmodulin (CaM) to the enzyme. However, the
9 conditions under which CaM is bound appear to be different
10 for iNOS, at least insofar as calcium concentration is
11 concerned. These three NOS enzymes have been intensively
12 studied, and the field has been recently reviewed; see for
13 example Michel and Feron (1997); Harrison (1997); and Mayer
14 and Hellens (1997). Although it was known from earlier
15 studies that eNOS could be multiply phosphorylated, the
16 mechanism of these phosphorylation events, including the
17 enzyme responsible for phosphorylation, and the role of
18 phosphorylation in modulation of eNOS function was not
19 known.

20 AMP-activated protein kinase (AMPK) is a
21 metabolic stress-sensing protein kinase which is known to
22 play an important role in the regulation of acetyl-CoA
23 carboxylase, leading to the acceleration of fatty acid
24 oxidation during vigorous exercise or ischaemia. AMPK is
25 well known as a regulator of lipid metabolism, and in
26 particular is known to have a role in cholesterol
27 synthesis, as reviewed in Hardie and Carling (1997). The
28 AMPK is also considered to play an important role in
29 exercise-enhanced glucose transport (Hayashi et al., (1998)
30 which is distinct from the insulin-mediated glucose uptake
31 mechanism. AMPK has mainly been studied in the liver,
32 heart and skeletal muscle. AMPK has been purified, and the
33 genes encoding the enzyme subunits were cloned (See
34 International Patent Applications

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1 numbers PCT/GB94/01093 and PCT/US97/00270 and publication
2 WO97/25341).

3 The mammalian AMPK (Mitchelhill et al, 1994) is
4 related to the *Saccharomyces cerevisiae* SNF1 protein
5 kinase. It is required for the expression of glucose-
6 repressed genes in response to nutritional stress which
7 requires growth on alternative carbon sources (Celenza and
8 Carlson, 1986); both the mammalian and yeast kinases are
9 activated by upstream kinases (Hardie and Carling, 1997).
10 The AMPK is involved in metabolic stress responses through
11 phosphorylation at Ser-79 and concomitant inhibition of
12 acetyl-CoA carboxylase and HMG-CoA reductase (Hardie and
13 Carling, 1997). Multiple AMPK isoforms occur. They
14 comprise $\alpha\beta\gamma$ heterotrimers consisting of either $\alpha 1$ or $\alpha 2$
15 catalytic sub-units (Stapleton et al, 1996; Stapleton et
16 al, 1997a), together with the non-catalytic subunits β and
17 γ (Mitchelhill et al, 1994; Carling et al, 1994; Stapleton
18 et al, 1994), which are related to the yeast *sipl1* and
19 *snf4p* respectively.

20 The AMPK $\alpha 2$ sub-unit gene is on chromosome 1
21 (Beri et al., 1994), the $\alpha 1$ sub-unit gene is on
22 chromosome 5, the $\beta 1$ and $\gamma 1$ sub-unit genes are on
23 chromosome 12, the $\beta 2$ sub-unit gene is on chromosome 1, and
24 the $\gamma 2$ sub-unit gene is localised on chromosome 7
25 (Stapleton et al, 1997). A $\gamma 3$ gene has been detected using
26 an expressed sequence tag (EST) generated by genome
27 sequencing (Accession No AA178898).

28 One of the genes encoding eNOS is on
29 chromosome 7, close to the gene for the $\gamma 2$ sub-unit of
30 AMPK. Another gene encoding nNOS is found on
31 chromosome 12. (The human gene map; SEE
32 <http://www.ncbi.nlm.nih.gov/cgi->

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1 bin/SCIENCE96/tsrch?QTEXT=nitric+oxide+synthase)
2 Recent work has shown that the AMPK in cardiac
3 and skeletal muscle is activated by vigorous exercise or by
4 ischaemic stress (Winder and Hardie, 1996; Vavvas et al,
5 1997; Kudo et al, 1995). This led us to investigate the
6 localization of the AMPK isoforms in these tissues. The
7 AMPK- α 2 isoform is present in capillary endothelial cells
8 in cardiac and skeletal muscle, and the AMPK- α 1 isoform
9 occurs in cardiac myocytes and vessels. The presence of
10 AMPK in endothelial cells led us to test bacterially-
11 expressed eNOS as a substrate, and we found that it is
12 readily phosphorylated by either AMPK- α 1 or AMPK- α 2.

13 We have now surprisingly found that the
14 AMP-activated protein kinase phosphorylates and regulates
15 endothelial NO synthase. We find that the AMPK
16 phosphorylates eNOS at two sites. In the presence of
17 calcium and calmodulin, Ser-1177 in the human sequence, and
18 Ser-1179 for the bovine sequence is phosphorylated in the
19 COOH-terminal tail of the enzyme, causing activation of
20 eNOS by shifting the calmodulin-dose dependence. In the
21 absence of added calcium and calmodulin, phosphorylation
22 also occurs at Thr-495 in the eNOS calmodulin-binding
23 sequence, and inhibits the enzyme. Ischaemia of the heart
24 causes activation of the AMPK and of eNOS, mimicking the
25 effects of phosphorylation at Ser-1177. Phosphopeptide-
26 specific antibodies to phosphorylated Ser-1177 were used to
27 confirm that this site was phosphorylated during ischaemia.
28 Our results are of special interest because they identify a
29 link between metabolic stress, which reduces ATP and
30 increases AMP, and signalling through eNOS to control
31 nutrient availability (via arterial vasodilation) as well
32 as suppressing myocardial contraction. This couples the
33 metabolic status of endothelial cells and myocytes with the

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1 vascular supply and mechanical demands. Our results
2 provide a new insight into the post-translational
3 regulation of eNOS which is of particular significance for
4 the cardiovascular and skeletal muscle field. In addition,
5 similarities in structure and behaviour between eNOS and
6 nNOS have been identified, enabling us to identify
7 modulators of the activity of both these enzymes.

8

9 SUMMARY OF THE INVENTION

10 According to a first aspect, the invention
11 provides a method of identifying modulators of AMPK-
12 mediated activation of a nitric oxide synthase enzyme
13 selected from the group consisting of eNOS, nNOS and nNOS μ ,
14 comprising the step of testing the ability of putative
15 modulators to increase or decrease phosphorylation of the
16 enzyme; said increase or decrease depending on the
17 calmodulin and calcium ion concentrations.

18 Preferably the specific phosphorylation of
19 Ser-1177 is assessed in the presence of calcium and
20 calmodulin.

21 In an alternative aspect, the invention provides
22 a method of identifying modulators of AMPK-mediated
23 inhibition of eNOS, comprising the step of testing a
24 putative modulator for its ability to decrease or increase
25 AMPK-mediated phosphorylation of eNOS in the presence of
26 limiting calcium ions. Preferably specific phosphorylation
27 of Thr-495 is assessed.

28 Compounds able to increase phosphorylation of
29 Ser-1177 or decrease phosphorylation of Thr-495 are
30 referred to herein as activators, and compounds able to
31 decrease phosphorylation of Ser-1177 or increase
32 phosphorylation of Thr-495 are referred to as inhibitors.

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1 In both aspects of the invention, one or more of
2 the following activities may optionally be additionally
3 assessed for each putative activator or inhibitor
4 identified by the method of the invention:

5 (a) Effect on smooth muscle contraction;
6 (b) Effect on inotropic activity of the
7 heart;
8 (c) Effect on chronotropic activity of the
9 heart; and
10 (d) Effect on platelet function.

11 It is expected that because the phosphorylation
12 site equivalent to Thr-495 in the eNOS calmodulin-binding
13 site is absent from the neuronal form of NOS, inhibitors
14 and activators identified by the method of the invention
15 will have at least some degree of tissue specificity.

16 Compounds that activate the AMP-activated protein
17 kinase are expected to be useful in ischaemic heart disease
18 by promoting both glucose and fatty acid metabolism, as
19 well as by increasing NOS activity to improve nutrient and
20 oxygen supply to the myocytes and to reduce mechanical
21 activity. These compounds would also have utility in
22 pulmonary hypertension and in obstructive airways disease.

23 For the purposes of this specification it will be
24 clearly understood that the word "comprising" means
25 "including but not limited to", and that the word
26 "comprises" has a corresponding meaning.

27

28 BRIEF DESCRIPTION OF THE FIGURES

29 Figure 1 shows immunofluorescence localization of
30 AMPK- α 2 in the heart and in the tibialis anterior muscle.

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1 Panel A shows a negative control section of rat
2 heart stained with control rabbit IgG and control mouse
3 IgG, together with anti-rabbit-FITC and anti-mouse-Texas
4 Red.

5 Panel B shows a section of rat heart stained with
6 affinity-purified rabbit polyclonal antibody against
7 AMPK- α 2 (491-514) and anti-rabbit-FITC.

8 Panel C shows the same section as Panel B,
9 stained with a monoclonal antibody against rat endothelium
10 recA-1 and anti-mouse-Texas Red.

11 Panel D shows the overlay of Panels B and C.
12 Colocalization can be seen by the coincidence of staining.
13 The arrows highlight specific endothelial cells that are
14 stained by both antibodies.

15 Panel E shows a negative control section of rat
16 tibialis anterior muscle stained with control rabbit IgG
17 and control mouse-IgG, together with anti-rabbit-FITC and
18 anti-mouse-Texas Red.

19 Panel F shows a section stained with affinity-
20 purified rabbit polyclonal antibody against AMPK- α 2
21 (491-514) and anti-rabbit-FITC.

22 Panel G shows the same section as in Panel B,
23 stained with a monoclonal antibody against rat endothelium
24 recA-1 and anti-mouse-Texas Red.

25 Panel H shows the overlay of Panels E and F.
26 Colocalization can be seen by the coincidence of staining.

27 Figure 2 illustrates phosphorylation of
28 recombinant eNOS by AMPK.

29 Top panel: eNOS was incubated with rat liver
30 AMPK- α 1 and [γ -³²P] ATP.

31 Lane 1: Coomassie-stained SDS-PAGE;

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1 Lane 2: Autoradiograph.

2 Lower panel: ^{32}P -tryptic phosphopeptide map of
3 eNOS.

4 Figure 3 shows the effect of phosphorylation of
5 eNOS by the AMPK with or without added Ca^{2+} -CaM. Rat
6 heart eNOS purified by 2',5'-ADP-Sepharose affinity
7 chromatography was phosphorylated by AMPK in the presence
8 of 0.8 μM CaM/3.2 μM Ca^{2+} (closed circles), in the absence
9 of Ca^{2+} -CaM (closed triangles) and without AMPK (open
10 squares). After phosphorylation, samples were diluted and
11 eNOS activity was measured. The lower panels show
12 phosphopeptide maps for rat heart eNOS phosphorylated in
13 the presence and absence of added Ca^{2+} -CaM.

14 Figure 4 shows the effect of ischaemia on the
15 activities of AMPK- α 1, AMPK- α 2 and eNOS.

16 Panel A shows the results of immunoprecipitation
17 using antibody specific for AMPK- α 1 and AMPK- α 2, assayed
18 using the SAMS peptide substrate. Results shown are mean \pm
19 SEM for n=5.

20 Panel B shows eNOS activity measured at 500 nM
21 CaM.

22 Panel C shows eNOS activities with full CaM-dose
23 responses for a representative experiment. Ischaemia time
24 points: 0 min (open squares), 1 min (closed diamonds),
25 10 min (closed circles) and 20 min (open triangles). The
26 results of 4 replicates were the same, except that in one
27 case the 20 min ischaemia eNOS CaM-dependence remained the
28 same as for 10 min.

29 Figure 5 shows a comparison of NOS sequences.
30 Phosphorylation site sequences for eNOS and nNOS are
31 indicated in a schematic model of NOS. Sequences from the
32 CaM-binding region (around the Thr-495 phosphorylation site

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1 in eNOS) and for the COOH-terminal tail (around the
2 Ser-1177 phosphorylation site in eNOS) are shown.

3 Figure 6 shows the effect of treatment of bovine
4 aortic endothelial cells with phorbol ester (PMA) and
5 okadaic acid on eNOS activity (upper pane) and the
6 phosphorylation at Ser-1177 and Thr-495 (lower panel).

7 Figure 7 shows the effect of treatment of bovine
8 aortic endothelial cells with 3-isobutyl-1-methylxanthine
9 (IBMX) and calyculin A on the phosphorylation at Ser-1177
10 and Thr-495.

11 Figure 8 shows a summary illustration of the
12 regulation of eNOS by phosphorylation at Thr-495 and Ser-
13 1177, mediated by protein kinases PKC, AMPK and Akt.
14 Reversal of the phosphorylation at these sites is mediated
15 by protein phosphatases PP1 and PP2A in response to
16 treating the cells with IBMX and PMA respectively.

17 Figure 9 shows the effect of a 30 second bicycle
18 sprint exercise on nNOS phosphorylation in human muscle.
19 The nNOS was extracted from biopsy material and probed for
20 phosphorylation at Ser-1417 using an anti-phosphopeptide
21 antibody. The left panel shows an immunoblot, and the
22 right panel shows quantitative analysis of 5 individuals.

23

24 DETAILED DESCRIPTION OF THE INVENTION

25 The invention will now be described in detail by
26 way of reference only to the following non-limiting
27 examples and to the figures.

28 We have surprisingly found that in the presence
29 of Ca^{2+} -calmodulin (CaM) eNOS is phosphorylated by AMPK at
30 Ser-1177, resulting in activation, whereas phosphorylation
31 of eNOS in the absence of Ca^{2+} occurs predominantly at
32 Thr-495, a site in the CaM-binding sequence, resulting in

- 10 -

1 inhibition. It had previously been considered that
2 phosphorylation was solely inhibitory. We have also found
3 that ischaemia of the heart leads to rapid activation of
4 both isoforms of the metabolic stress-sensing enzyme AMPK
5 and eNOS. These data suggest that the AMPK may operate an
6 "inside-out" signalling pathway that leads to arterial
7 vasodilation and reduced myocardial contraction, so
8 coupling the metabolic status of endothelial cells and
9 myocytes with the vascular supply and mechanical activity.

10

11 Example 1 Immunofluorescence Localisation of AMPK- α 2
12 in Heart and Skeletal Muscle

13 Confocal immunofluorescence microscopy using
14 affinity-purified rabbit polyclonal antibody directed
15 against AMPK- α 2 (antibody 491-414. Staining with
16 fluorescence-labelled anti-rabbit antibody showed that the
17 α 2 isoform is found predominantly in capillary endothelial
18 cells in both cardiac muscle and skeletal muscle, while
19 cardiac myocytes and blood vessels showed intense but
20 diffuse staining for the α 1 AMPK isoform. In skeletal
21 muscle, the α 2 isoform was found in endothelial cells of
22 capillaries, and in fast-twitch muscle fibres, whereas the
23 α 1 isoform was found in Type I aerobic fibres.

24 Localisation of AMPK- α 2 in capillary endothelial cells in
25 both cardiac and skeletal muscle is illustrated in
26 Figure 1.

27

28 Example 2 AMPK Phosphorylates Recombinant eNOS

29 Bacterially expressed eNOS, coexpressed with CaM
30 by the method of Rodriguez-Crespo *et al* (1996), was
31 phosphorylated by either AMPK- α 1, as shown in Figure 2 top

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1 panel, or AMPK- α 2. Recombinant eNOS phosphorylation by
2 immunoprecipitated AMPK- α 2 was detected. Since we have
3 been unable to purify high specific activity AMPK- α 2, no
4 further characterisation of eNOS regulation or the sites of
5 phosphorylation by the α 2 isoform was undertaken. Analysis
6 of the phosphorylation sites in eNOS following tryptic
7 digestion revealed four phosphopeptides generated from
8 three separate sites (Figure 2 bottom panel, A, A', B, C).
9 Identification of phosphorylation sites by mass
10 spectrometry and Edman sequencing, using the modified
11 method described by (Mitchelhill and Kemp, 1999), revealed
12 that Ser-1177 was the most prominent phosphorylation site,
13 as shown in Figure 2 bottom panel, A, A', and that its
14 phosphorylation was dependent on the presence of Ca^{2+} -CaM.

15 Phosphopeptide isolation from in-gel tryptic
16 digests was carried out as described by Mitchelhill *et al*
17 (1997a). Greater than 98% of the radioactivity was
18 recovered from the gel. Peptides isolated and characterized
19 by mass spectrometry and Edman sequencing are set out in
20 Table 1.

Table 1
Phosphopeptides Isolated from In-Gel tryptic Digests

Observed Mass	Phosphopeptide	Sequence	Calculated Mass
1440.0	B	KKTFKEVANAVK	1361.1 (*1441.7)
1174.1	A	TQXFSLQER	1094.5 (*1174.5)
1445.6	A'	IRTQXFSLQER	1363.7 (*1443.7)
1176.7	C	PCLGSLVFPF	1095.6 (*1175.6)

10

where: "pc" denotes pyridylethyl cysteine.

• denotes calculated mass of mono-phosphorylated peptide.

15

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1 The location of the phosphorylation site in
2 peptide A, TQXFSLQER, was identified by 32 P-phosphate
3 release sequencing (Mitchell et al, 1997a). eNOS
4 phosphorylated by the AMPK- α 1 was no longer recognized by
5 the antibody to the eNOS COOH-terminal tail; nor was it
6 eluted from the ADP-Sepharose affinity column by
7 100 mM NADPH. These properties prevented the direct
8 confirmation of Ser-1177 phosphorylation *in situ*. This is
9 illustrated in Venema et al, 1996.

10 A second site, Thr-495, was phosphorylated in the
11 absence of Ca^{2+} -CaM or when EGTA was present. This is
12 illustrated in Figure 2 bottom panel, B. This residue is
13 located in the CaM-binding sequence,

14 TRKKT⁴⁹⁵FKEVANAVKISASLM,

15 between the oxidase and reductase domains of eNOS (Venema
16 et al, 1996). Ser-101 in the N-terminal region of eNOS was
17 identified as a minor site of phosphorylation (Figure 2
18 bottom panel, C).

19 Synthetic peptides containing Thr-495 or Ser-1177
20 were readily phosphorylated by AMPK, with similar kinetic
21 values to the SAMS peptide substrate. The peptide
22 containing Thr-495, GTGITRKKTFKEVANAVK, was phosphorylated
23 with a K_m of $39 \pm 10 \mu\text{M}$ and a V_{max} of
24 $6.7 \pm 0.6 \mu\text{mol/min/mg}$, whereas the peptide containing
25 Ser-1177, RIRTQSFSLQERQLRG was phosphorylated with a K_m of
26 $54 \pm 6 \mu\text{M}$ and a V_{max} of $5.8 \pm 0.3 \mu\text{mol/min/mg}$. These are
27 comparable to results obtained using the well-characterized
28 SAMS peptide substrate, which has a K_m $33 \pm 3 \mu\text{M}$ and a V_{max}
29 of $8.1 \pm 1.5 \mu\text{mol/min/mg}$ (Michell et al, 1996). The *in*
30 *vitro* phosphorylation of the peptides confirms the
31 identification sites of phosphorylation.

- 14 -

1 Example 3

2 Effect of Ca^{2+} -CaM on Phosphorylation of
2 eNOS by AMPK

3 The eNOS activity was determined by measuring
4 $\text{L-[}^3\text{H]citrulline}$ production, using the method of Balligand
5 et al, 1995. The recombinant eNOS was coexpressed with
6 CaM, as described by Rodriguez-Crespo and Ortiz de
7 Montellano, 1996. Partially purified rat heart eNOS
8 contained some Ca^{2+} -CaM. In the absence of added EGTA, CaM
9 dependence was observed at 0-100 nM added CaM. In order to
10 investigate the changes in NOS activity with
11 phosphorylation in the absence and presence of Ca^{2+} -CaM,
12 EGTA buffering was used to achieve CaM dose response curves
13 in the range 0-1 μM . Routinely, 7-15 μM EGTA was added to
14 make eNOS activity dependent upon added CaM. Where
15 Ca^{2+} -CaM was used in the phosphorylation reaction prior to
16 eNOS assay, the samples were either diluted so that the
17 extra Ca^{2+} -CaM was negligible, or the indicated
18 concentrations represent total final concentrations of
19 added Ca^{2+} -CaM.

20 Cardiac eNOS was partially purified as follows.
21 Twenty rat hearts were homogenised in 80 ml of ice-cold
22 buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA,
23 1 mM DTT, 50 mM NaF, 5 mM Na Pyrophosphate,
24 10 $\mu\text{g/ml}$ Trypsin inhibitor, 2 $\mu\text{g/ml}$ Aprotinin,
25 1 mM Benzamidine, 1 mM PMSF, 10% Glycerol, 1% Triton-X-
26 100]. The homogenate was put on ice for 30 min and
27 centrifuged at 16,000 $\times g$ for 30 min. The supernatant was
28 incubated with 2 ml of 2',5'-ADP-Sepharose (Bredt and
29 Snyder, 1990). The suspension was incubated for one hour
30 before washing in a fritted column, with 20 ml of buffer A
31 and 20 ml of buffer A containing 0.5 M NaCl, and then with
32 20 ml of buffer B [50 mM Tris-HCl, pH 7.5, 1 mM DTT,
33 10% Glycerol, 0.1% Triton-X-100]. eNOS was eluted with
34 buffer B containing 2 mM NADPH, then subjected to

- 15 -

1 centrifugal filtration (ULTRAFREE-MC MILLIPORE) to remove
2 NADPH. Immunoblotting was used for selective detection of
3 eNOS rather than nNOS.

4 Phosphorylation of eNOS by AMPK in the presence
5 of Ca^{2+} -CaM resulted in activation, but CaM-dependence was
6 retained, as shown in Figure 3 top panel. Activation
7 shifted the dose response curve for CaM to the left.
8 Phosphopeptide mapping revealed that activation of eNOS was
9 correlated with phosphorylation of Ser-1177 but not of Thr-
10 495, as shown in Figure 3 lower panel. Phosphorylation
11 without added Ca^{2+} -CaM enhanced Thr-495 phosphorylation,
12 suppressed Ser-1177 phosphorylation, and inhibited eNOS
13 activity (Figure 3 top panel). The inhibition of eNOS
14 activity by Thr-495 phosphorylation is consistent with
15 earlier reports that phosphorylation of synthetic peptides
16 corresponding to this region by protein kinase C inhibits
17 CaM-binding (Matsubara *et al.*, 1996). Similar results have
18 been reported for nNOS (Loche *et al.*, 1997).

19

20 Example 4 Effect of Ischaemia on Activities of
21 AMPK- α 1, AMPK- α 2 and eNOS

22 Langendorf preparations of isolated perfused rat
23 heart were subjected to ischaemia according to the method
24 of Kudo *et al* (1995). AMPK- α 1 and AMPK- α 2 isoforms were
25 immunoprecipitated using α 2 (490-516) or α 1 (231-251)
26 antibodies, and assayed using the SAMS peptide substrate
27 (Michell *et al*, 1996; Hardie and Carling, 1997). eNOS
28 activity was measured as described in Example 3. The
29 results are shown in Figure 4. Both α 1 and α 2 isoforms are
30 activated, as shown in Figure 4A, indicating that AMPK is
31 activated in both capillary endothelial cells, which have
32 predominantly the α 2 isoform, and in cardiac myocytes,

- 16 -

1 which have predominantly the $\alpha 1$ isoform. AMPK activation
2 during ischaemia is also accompanied by eNOS activation and
3 changes in the CaM dependence, as shown in Figures 4B and
4 4C, mimicking the effect of eNOS phosphorylation by AMPK *in*
5 *vitro*, as shown in Figure 3.

6 Polyclonal antibodies were raised against
7 synthetic phosphopeptides based on the eNOS sequence:
8 RIRTQSpFSLQER and GITRKKTpFKEVANCV. Rabbits were immunized
9 with phosphopeptides coupled to keyhole limpet haemocyanin
10 and then emulsified in Freund's complete adjuvant, using
11 conventional methods. The antibodies were purified using
12 the corresponding phosphopeptide affinity columns after
13 thorough preclearing with dephosphopeptide affinity
14 columns. The specificity of the purified antibodies was
15 confirmed using both EIA and immunoblotting, confirming
16 that they did not recognize recombinant dephospho-eNOS.

17 Using the anti-phosphopeptide antibodies to Ser-
18 1177 and Thr-495 phosphorylation sites we observed that
19 phosphorylation of Ser-1177 was increased approximately 3-
20 fold by ischaemia, but that there was no detectable change
21 in the Thr-495 phosphorylation under these conditions.
22 Heart muscle contains eNOS in both capillary endothelial
23 cells and cardiac myocytes (Balligand *et al*, 1995), with
24 low levels of the nNOS μ isoform (Silvagno *et al*, 1996).

25 The sequences of the three types of NOS are
26 compared in Figure 5, which shows the CaM-binding region
27 and the C-terminal tail. In nNOS Ser-1417 corresponds to
28 eNOS Ser-1177, whereas iNOS is truncated, and has a Glu in
29 this region. Both iNOS and nNOS lack a phosphorylatable
30 residue equivalent to Thr-495 in the CaM-binding region.

1

2

3 **Example 5 Effect of Stimulation of Protein Kinase C on**
4 **eNOS Phosphorylation**

5 Bovine aortic endothelial cells cultured in 0.1%
6 foetal calf serum for 20 hours (serum starved) were
7 subjected to treatment with the protein kinase C activator
8 0.1 μ M phorbol-12-myristate-13-acetate (PMA) for 5 min.
9 PMA treatment increased the phosphorylation of eNOS at Thr-
10 495 and decreased the phosphorylation at Ser-1177, as
11 measured using anti-phosphopeptide specific antibodies.
12 The antibodies used were the same as those described in
13 Example 4. The results are shown in Figure 6. In cells
14 cultured in medium without calcium we observed a 4-fold
15 decrease in Ser-1177 phosphorylation. Furthermore, when
16 cells were incubated in standard medium containing calcium
17 addition of the calcium ionophore A23187 (10 μ M for 90
18 seconds) increased Ser-1177 phosphorylation by a further 7-
19 fold. Preincubation of the cells with 0.5 μ M okadaic acid
20 prevented the dephosphorylation of Ser-1177 by PMA
21 treatment, and greatly augmented the phosphorylation of
22 Thr-495 (Results mean \pm SEM, n =6). Since okadaic acid
23 inhibits protein phosphatase PP2A, the results indicate
24 that PP2A is responsible for dephosphorylation of Ser-1177.
25 The changes observed in Thr-495 and Ser-1177
26 phosphorylation in response to treatment with PMA and
27 okadaic acid were reflected in the activity of eNOS.
28 Increased phosphorylation of Thr-495 with PMA or PMA plus
29 okadaic acid was associated with reduced eNOS activity.
30 Okadaic acid alone increased Ser-1177 phosphorylation
31 without altering Thr-495 phosphorylation, and was
32 associated with increased eNOS activity (Figure 6 upper
33 panel).

1

2

3 Example 6 Effect of Inhibition of Phosphodiesterase
4 and Phosphatase on the Phosphorylation of eNOS

5 The experimental details were similar to those
6 for Example 5. Bovine aortic endothelial cells were
7 preincubated with or without 10 nM of the phosphatase
8 inhibitor calyculin A for 10 min, and then incubated with
9 or without 0.5 mM of the phosphodiesterase inhibitor, 3-
10 isobutyl-1-methylxanthine (IBMX) for 5 min. As shown in
11 Figure 7, IBMX treatment caused enhanced phosphorylation of
12 Ser-1177 and dephosphorylation of Thr-495. Preincubation
13 with calyculin A prevented the dephosphorylation of Thr-
14 495. (Results mean \pm SEM, n =6). Since calyculin A
15 inhibits protein phosphatase PP1, the results indicate that
16 PP1 is responsible for dephosphorylation of Thr-495.

17

18 DISCUSSION

19 Since the identification of the Ser-1177
20 phosphorylation site by the present inventors, it has been
21 recognized that other protein kinases phosphorylate at this
22 site. In particular, the protein kinase Akt (also named
23 PKB) phosphorylates Ser-1177 in response to stimulation of
24 endothelial cells by vascular endothelial growth factor
25 (VEGF) (Fulton et al. 1999; Michell et al., 1999) or to fluid
26 shear stress (Dimmeler et al., 1999; Gallis et al., 1999).
27 In the study by Gallis et al. (1999) it was reported that
28 fluid shear stress stimulated the phosphorylation of Ser-
29 116 in the sequence KLQTRPSPGPPPA. Neither the kinase
30 responsible nor the functional effects of phosphorylation
31 of this site on eNOS has yet been identified. This
32 phosphorylation site is present in the oxidase domain.

- 19 -

1 We have found that phosphorylation of eNOS at
2 Thr-495 by protein kinase C occurs in endothelial cells
3 that have been serum starved and incubated in calcium-free
4 medium in the presence of the phorbol ester PMA. There is
5 a reciprocal relationship between phosphorylation at Ser-
6 1177 and Thr-495 in endothelial cells. Protein kinase C
7 phosphorylates both sites *in vitro*, but stimulation of
8 protein kinase C in endothelial cells with phorbol ester
9 causes enhanced Thr-495 phosphorylation but marked
10 phosphorylation of Ser-1177. The dephosphorylation of Ser-
11 1177 is prevented by okadaic acid but not by calyculin A,
12 indicating that phosphatase PP2A is responsible. Okadaic
13 acid also greatly enhances the phosphorylation of Thr-495
14 in response to phorbol ester. Thrombin, which also acts
15 via protein kinase C, stimulates phosphorylation of Thr-495
16 and dephosphorylation of Ser-1177.

17 In contrast, treatment of endothelial cells with
18 the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine
19 (IBMX) causes a pronounced dephosphorylation of eNOS at
20 Thr-495 and enhanced Ser-1177 phosphorylation.
21 Dephosphorylation of Thr-495 in response to IBMX is blocked
22 by treatment with calyculin A, suggesting that phosphatase
23 PP1 is responsible for Thr-495 dephosphorylation.

24 These relationships are summarised in Figure 8.
25 We find that exercise of skeletal muscle results in the
26 phosphorylation of nNOS μ at Ser-1417, the site
27 corresponding to Ser-1177 in eNOS (see Figure 5).
28 Electrical stimulation of rat extensor digitorum longus
29 (EDL) muscle was found to activate the AMPK, to
30 phosphorylate acetyl CoA carboxylase at Ser-79 (the
31 inhibitory site), and to phosphorylate nNOS μ at Ser 1417.
32 Similarly, in biopsies of human skeletal muscle following
33 vigorous exercise, such as a 30-second bicycle sprint,
34 there is a 10-fold increase in phosphorylation on Ser-79 in

- 20 -

1 acetyl CoA carboxylase and a 7.5-fold increase in nNOS μ
2 phosphorylation at Ser-1417 (see Figure 9).

3 Endothelial-derived NO has a critical role in
4 preventing premature platelet adhesion and aggregation that
5 leads to thrombus formation (Radomski and Moncada, 1993).
6 There is evidence that the protective effects of elevated
7 high-density lipoprotein (HDL) on the cardiovascular system
8 may be mediated via increased platelet NO production.
9 Apolipoprotein E, a component of HDL, acts on a receptor
10 (apoER2) present in platelets to stimulate the NO signal
11 transduction pathway (Riddell *et al.*, 1997; Riddell and
12 Owen, 1999).

13 Activation of eNOS by phosphorylation of its
14 COOH-terminal tail gives new insight into eNOS
15 autoinhibition. The increased activity and shift in the
16 CaM-dose dependence with phosphorylation at Ser-1177
17 suggest that in eNOS, and perhaps nNOS, the COOH-terminal
18 tails act as partial autoregulatory sequences analogous to
19 those in the CaM-dependent protein kinases (Kemp and
20 Pearson, 1991; Kobe *et al.*, 1996).

21 The COOH-terminal tail of eNOS is only fully
22 accessible to the AMPK when Ca^{2+} -CaM is bound, consistent
23 with this region being buried in the absence of CaM. As
24 can be seen from Figure 5, there is a high level of
25 similarity between eNOS and nNOS in their COOH-terminal
26 tails, whereas iNOS is distinct. It is known that the iNOS
27 CaM-binding, which is characterised by a low
28 Ca^{2+} -dependence, requires both the canonical CaM-binding
29 sequence and distal residues in the COOH-terminus that
30 cannot be satisfied by nNOS chimeras (Ruan *et al.*, 1996).
31 Without wishing to be bound by any proposed mechanism, we
32 believe that eNOS and nNOS are autoinhibited by their
33 COOH-terminal tails, requiring a two-stage activation

1 process for full activity with both CaM-binding and
2 phosphorylation in the tail, whereas iNOS requires only CaM
3 binding. Recently Salerno *et al* (1997) proposed that an
4 insert sequence in the FMN-binding domain may also be
5 important in autoregulation.

6 Previous studies have shown that eNOS may be
7 phosphorylated both *in vitro* and *in vivo*, but the precise
8 sites of phosphorylation and the function of the
9 phosphorylation events have not hitherto been fully
10 characterized (reviewed in Michel and Feron, 1997). eNOS
11 is the first example of an enzyme activated by AMPK to be
12 identified, and is also unusual because phosphorylation can
13 lead to either activation or inhibition, depending on the
14 availability of Ca^{2+} -CaM. Other enzymes, notably the
15 cyclin-dependent protein kinases, are activated or
16 inhibited by phosphorylation, but this is catalysed by
17 different protein kinases. Protein kinase C phosphorylates
18 Thr-495 in eNOS, demonstrating intersecting regulatory
19 pathways acting on eNOS by phosphorylation of Thr-495 or
20 Ser-1177. It is also possible that persistent activation
21 of protein kinase C, for example in response to
22 hyperglycaemia induced by diabetes, could chronically
23 suppress phosphorylation of eNOS at Ser-1177, and thereby
24 reduce its activity.

25 The regulation of eNOS by AMPK extends the
26 conceptual relationship between the yeast snf1p kinase and
27 the AMPK. Snf1p kinase modulates the supply of glucose from
28 the environment by secreting invertase whereas the
29 mammalian AMPK integrates metabolic stress signalling with
30 the control of the circulatory system. Thus intracellular
31 metabolic stress signals within endothelial cells and
32 myocytes can elicit improved nutrient supply and suppress
33 mechanical activity of the muscle.

- 22 -

1 It will be apparent to the person skilled in the
2 art that while the invention has been described in some
3 detail for the purposes of clarity and understanding,
4 various modifications and alterations to the embodiments
5 and methods described herein may be made without departing
6 from the scope of the inventive concept disclosed in this
7 specification.

8 References cited herein are listed on the
9 following pages, and are incorporated herein by this
10 reference.

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4

CLAIMS:

1. A method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOS μ , comprising
5 the step of testing putative modulators for their ability to increase or decrease phosphorylation of the enzyme, said increase or decrease depending on the calmodulin and calcium ion concentrations.
2. A method according to claim 1, in which the specific
10 phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.
3. A method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase
15 AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions.
4. A method according to claim 3, in which the specific phosphorylation of Thr-495 is assessed.
5. A method according to any one of Claims 1 to 4, in
20 which one or more of the following activities is additionally assessed:
 - (a) Effect on smooth muscle contraction;
 - (b) Effect on inotropic activity of the heart;
 - (c) Effect on chronotropic activity of the heart; or
 - 25 (d) Effect on platelet function.
6. A method according to any one of Claims 1 to 5, in which the modulator is an activator, as herein defined.
7. A method according to Claim 6, in which the activator promotes both glucose metabolism and fatty acid metabolism.

- 29 -

8. A method according to any one of Claims 1 to 5, in which the modulator is an inhibitor, as herein defined.
9. A method according to any one of Claims 3 to 8, in which the modulator acts preferentially on non-neuronal cells.
10. A method according to Claim 1 or Claim 2, in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.
11. A method according to Claim 3, in which the modulator promotes the dephosphorylation of Thr-495 and stimulates eNOS activity.
12. A method according to Claim 1 or Claim 2 in which the modulator promotes phosphorylation of nNOS or nNOS μ at Ser-1417.
13. A method according to Claim 1 or Claim 2 in which the modulator promotes dephosphorylation of nNOS or nNOS μ at Ser-1417.

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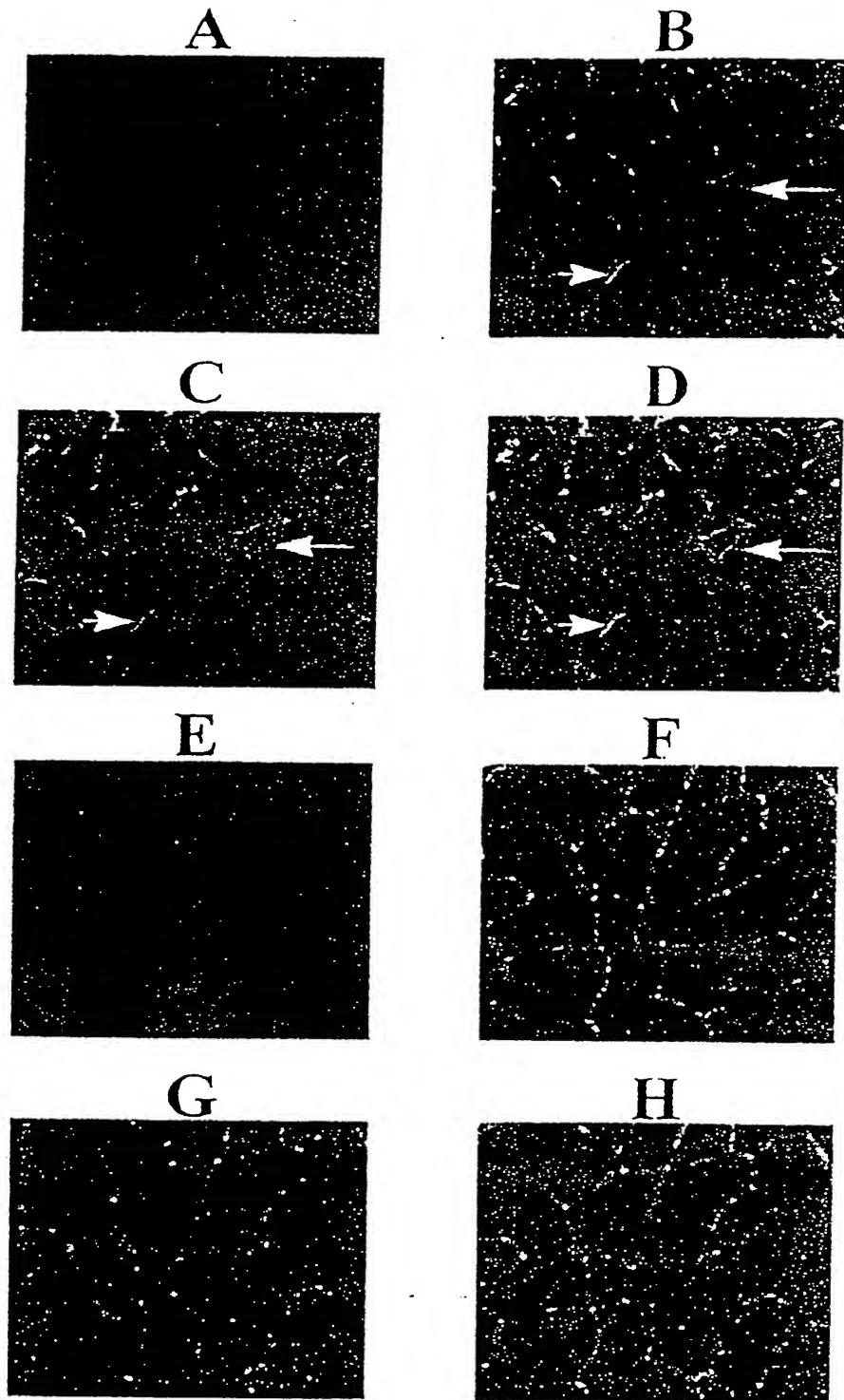


Figure 1

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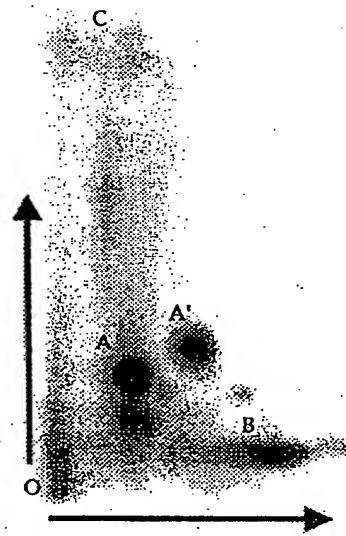
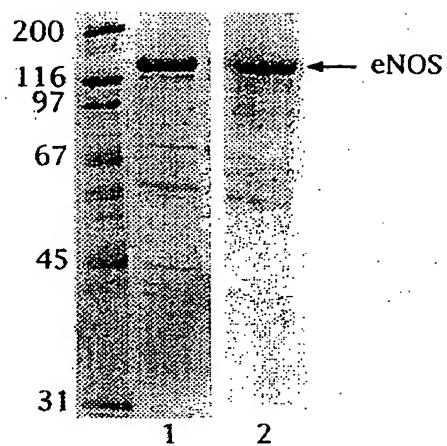
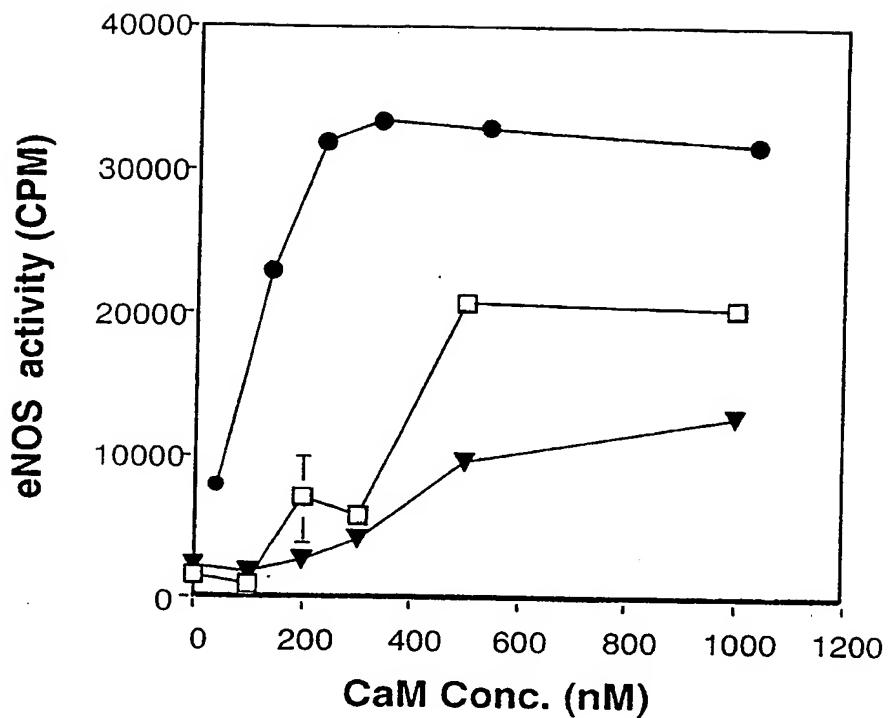


Figure 2

A

3/9



eNOS + AMPK
+ Ca^{2+} -CaM

A A B

Figure 3

4/9

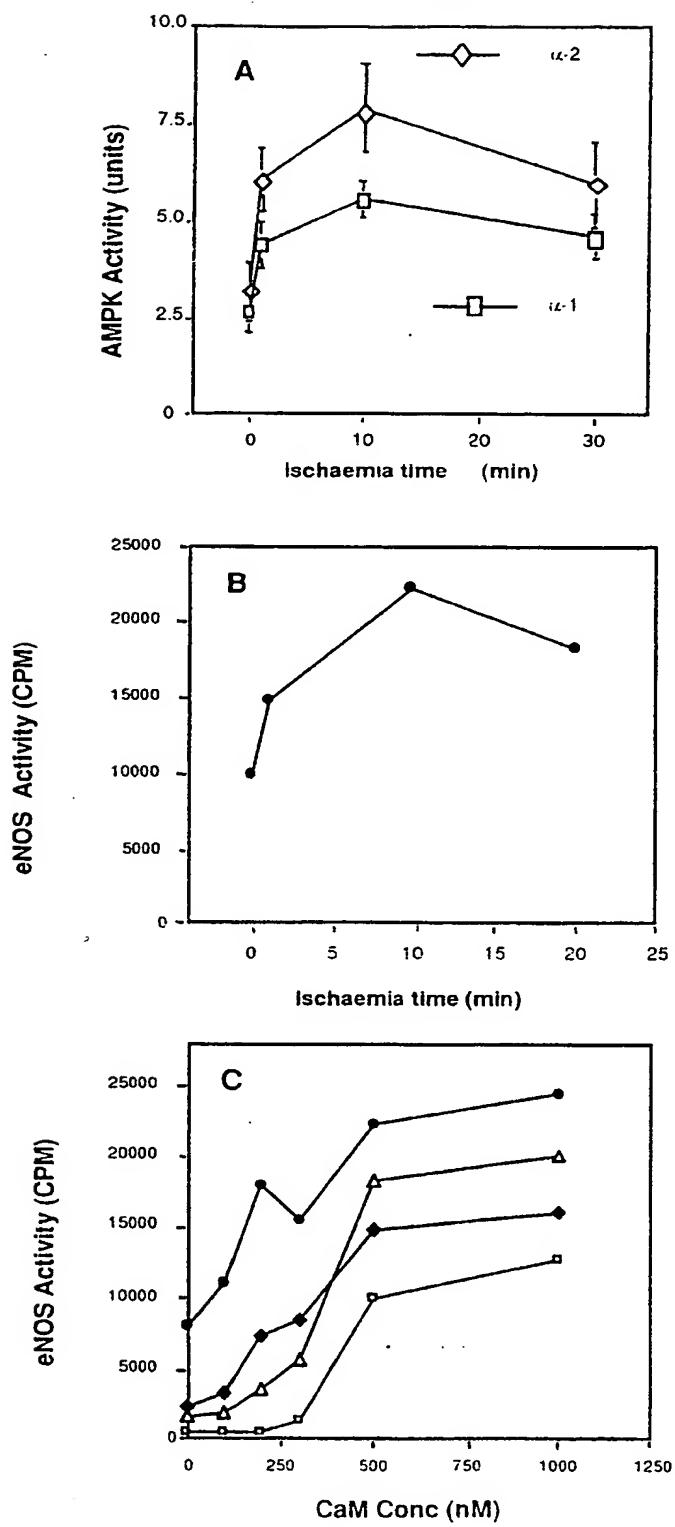


Figure 4

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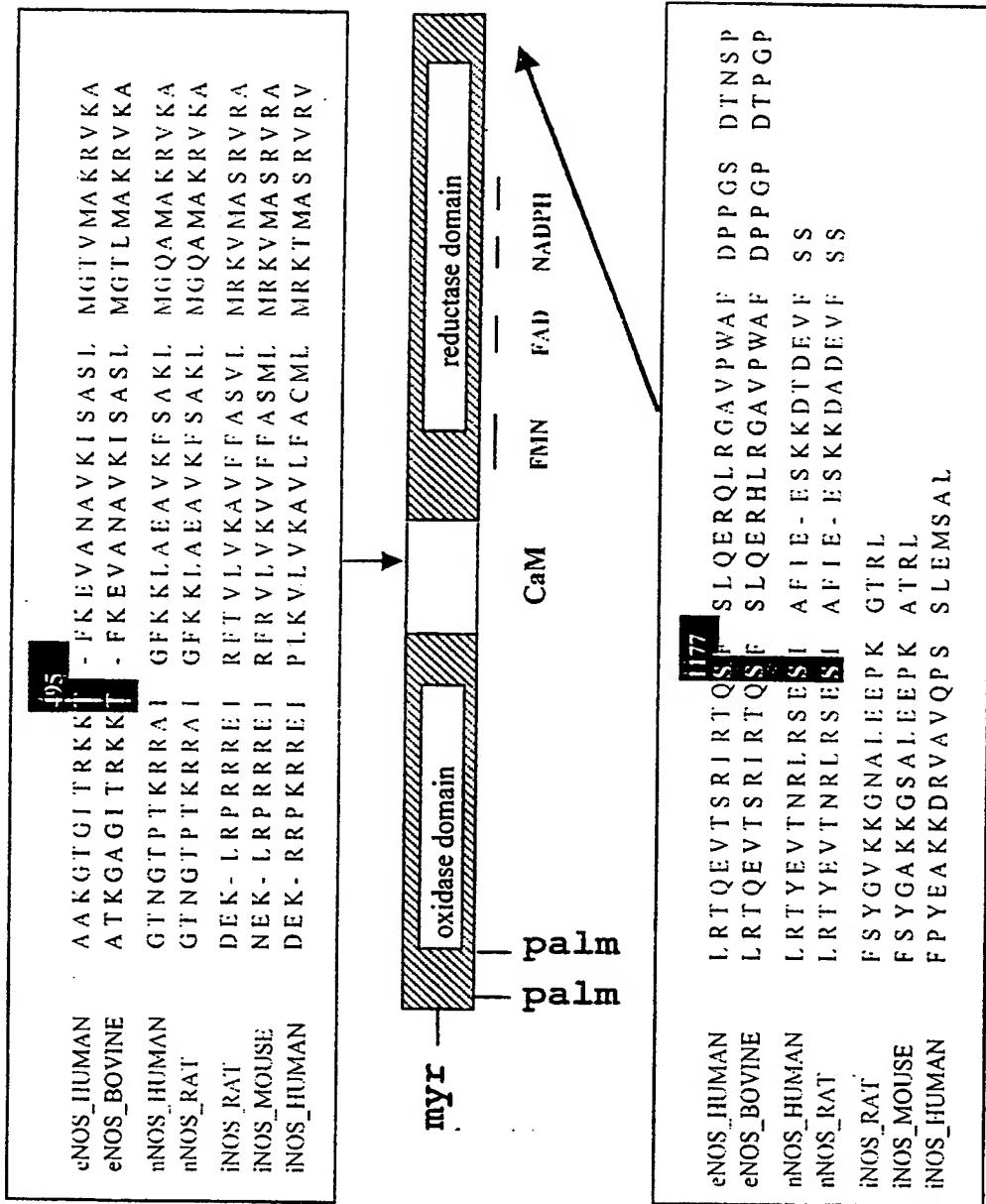


Figure 5

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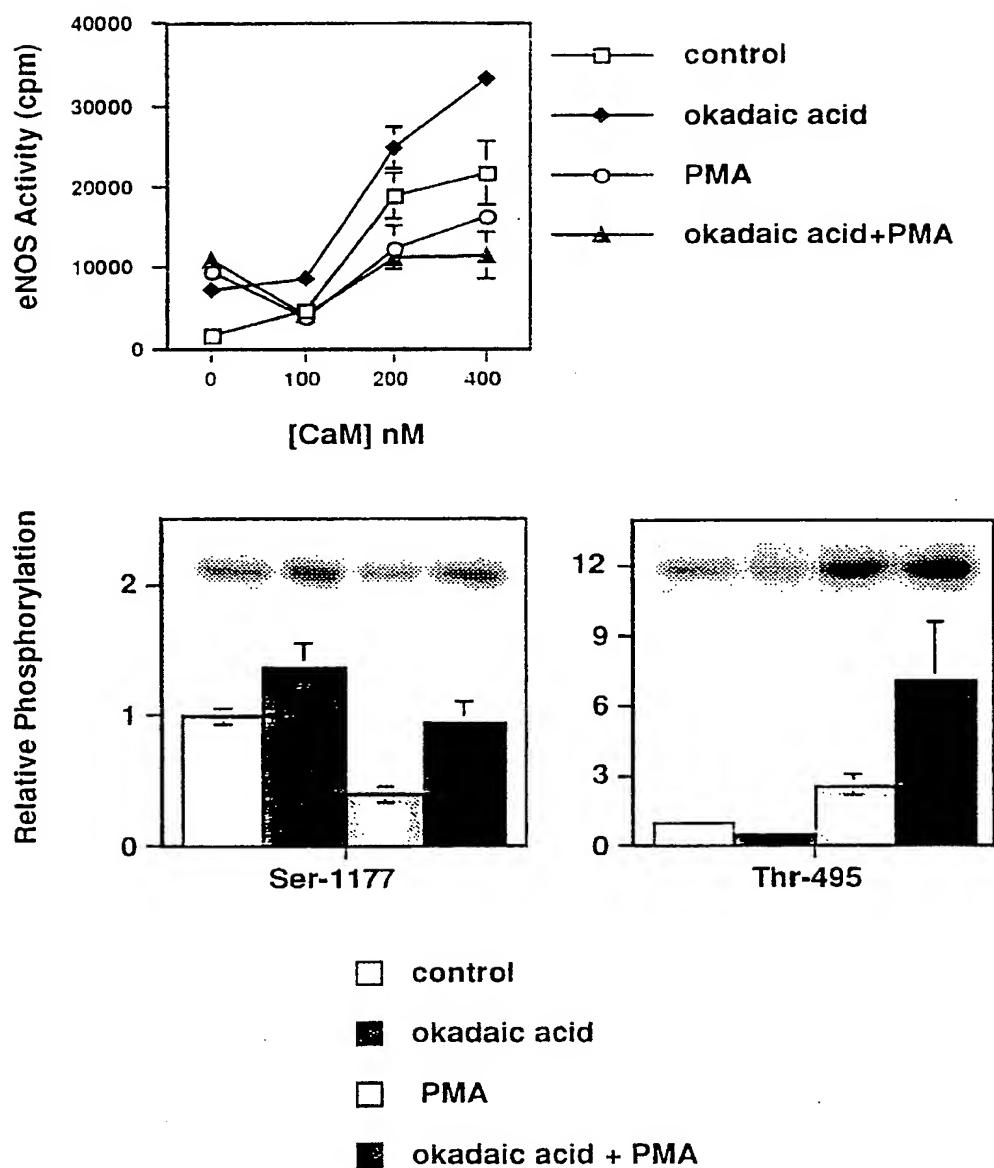


Figure 6

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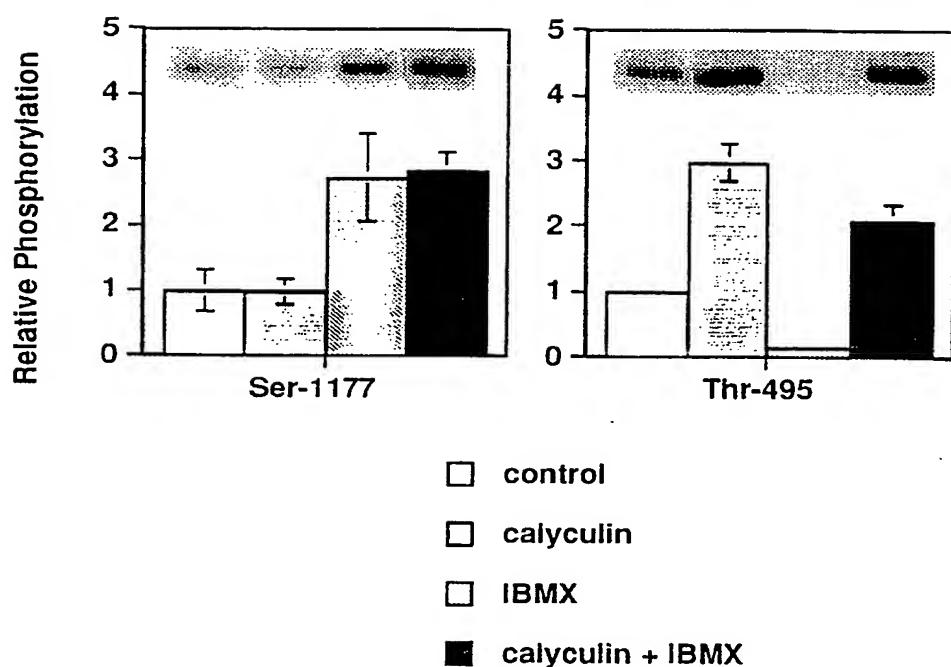
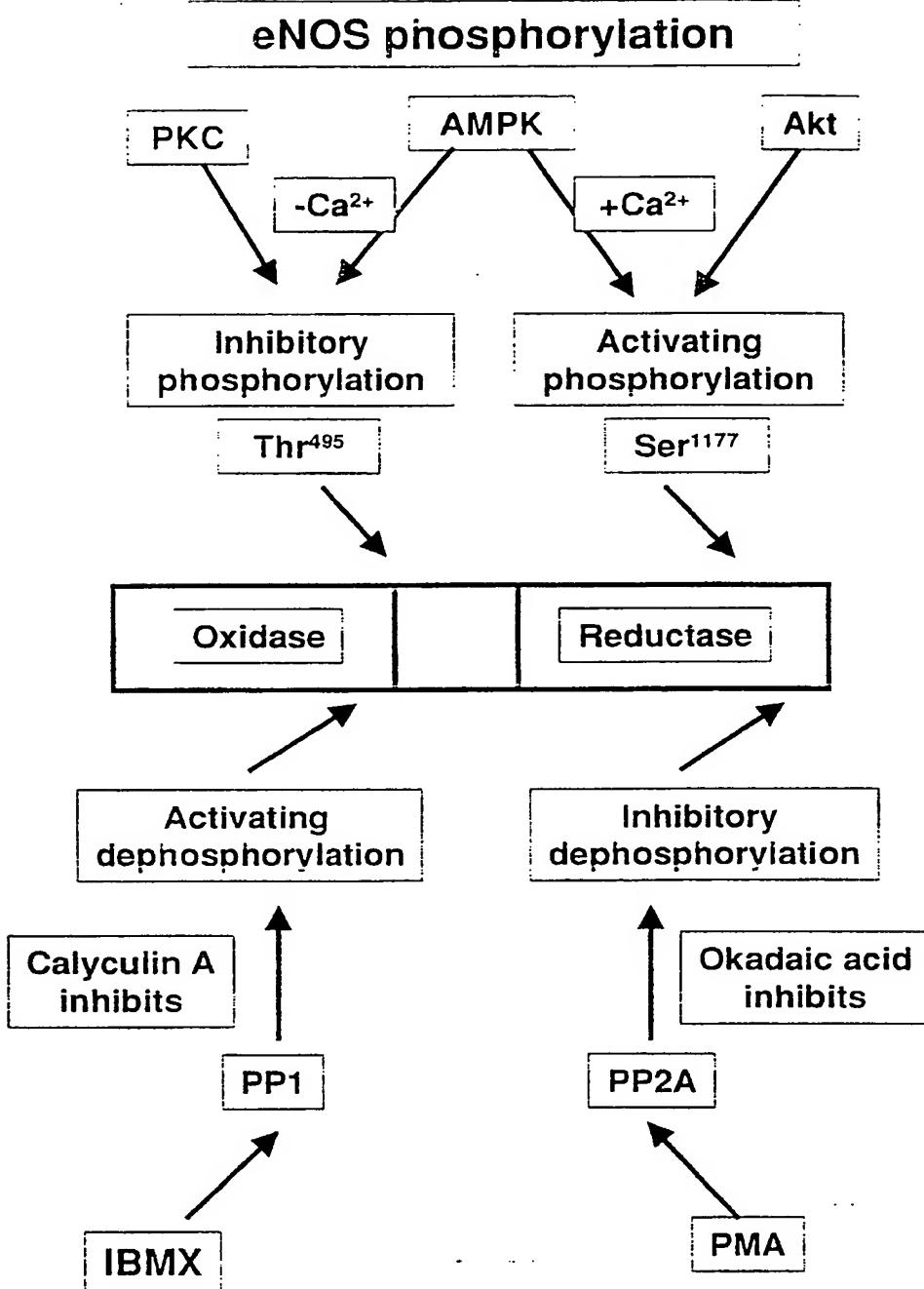


Figure 7

*Figure 8*

9/9



Relative fold

Figure 9

INTERNATIONAL SEARCH REPORT

International application No. PCT/AU 99/00968
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A. CLASSIFICATION OF SUBJECT MATTER																						
Int Cl ⁶ : C12Q 1/26, 1/27, 1/48																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols) IPC (WPAT - WORLD PATENTS INDEX), CHEMICAL ABSTRACTS																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched MEDLINE																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, Medline, Chemical Abstracts. Search terms: nitric oxide synthase, ampk, amp protein kinase:																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
P, X	FEBS Letters, volume 443, 1999, Chen et al., "AMP-activated protein kinase phosphorylation of endothelial NO synthase", pages 285 to 289	1 to 11																				
P, X	WO 99/26657 (MEDICAL UNIVERSITY OF SOUTH CAROLINA) 3 June 1999	1, 3, 5 to 9																				
A	The Journal of Biological Chemistry, volume 267, number 16, 1992, Bredt et al., "Nitric Oxide Synthase Regulatory Sites", pages 10976 to 10981	1 to 1																				
<input type="checkbox"/> Further documents are listed in the continuation of Box C		<input type="checkbox"/> See patent family annex																				
<p>* Special categories of cited documents:</p> <table> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
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"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 17 December 1999	Date of mailing of the international search report 21 DEC 1999																					
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer CHRISTOPHER LUTON Telephone No.: (02) 6283 2256																					

